Description

MODIFIED PROMOTER

Technical Field

[0001]

The present invention relates to a modified promoter DNA, an expression vector containing the DNA, a recombinant microorganism containing the expression vector, and a method for producing proteins or polypeptides through use of the recombinant microorganism.

Background of the Invention

[0002]

Microorganisms are widely used for industrially producing a broad range of useful substances, including alcoholic beverages, certain types of foods such as *miso* (i.e., fermented soybean paste) and *shoyu* (i.e., soy sauce), amino acids, organic acids, nucleic-acid-related substances, antibiotics, sugars, lipids, and proteins. These substances also find diversified uses, including foods, pharmaceuticals, detergents, products for daily use such as cosmetics, and a variety of chemical raw materials.

[0003]

In industrial production of useful substances by use of microorganisms, improvement of productivity is one major topic of interest, and one approach therefor is breeding of

microorganisms through mutagenesis or other genetic means. Recently, in particular, with advancement of microbial genetics and biotechnology, more efficient production of useful substances through gene recombination techniques attracts attention.

[0004]

Studies on promoters necessary for gene transcription have heretofore been actively conducted. For example, in relation to Bacillus subtilis, as a promoter region which is useful in achieving powerful transcription of a gene encoding a heterologous protein or polypeptide, there have been utilized a promoter region of an alkaline cellulase gene originating from Bacillus sp. KSM-64 (FERM BP-2886) (see, for example, Non-Patent Document 1) and a promoter region which is found in an upstream site of an alkaline cellulase gene originating from Bacillus sp. KSM-S237 (FERM BP-7875).

[0005]

However, in production on an industrial scale, reduction in production cost is necessary, and higher productivity is demanded, because the above-mentioned promoter regions currently in use do not necessarily promise sufficiently enhanced productivity.

Patent Document 1: JP-A-2000-210081

Non-Patent Document 1: Biosci. Biotech. Biochem., 59, 2172, (1995)

Disclosure of the Invention

[0006]

The present invention provides a promoter DNA which has been modified so that a nucleotide sequence containing a promoter recognized by SigA and bases in the vicinity thereof is recognized by SigA and SigE.

[0007]

The present invention also provides an expression vector containing the promoter DNA, a recombinant organism containing the expression vector, and a method of producing a protein or polypeptide characterized by culturing the recombinant microorganism.

[0008]

The present invention further provides a method of constructing a promoter DNA, characterized by modifying a nucleotide sequence containing a promoter recognized by SigA and bases in the vicinity thereof so that the promoter DNA is recognized by SigA and SigE.

Brief Description of the Drawings

[0009]

[Fig. 1] A schematic chart showing a procedure for introducing a SigE-recognized promoter sequence by means of SOE-PCR.

[Fig. 2] A schematic chart showing a method of constructing a plasmid for producing alkaline cellulase, wherein the plasmid has been modified so as to contain a SigE-recognized promoter sequence.

Mode for Carrying Out the Invention

[0010]

The present invention provides a modified promoter DNA capable of increasing the transcriptional amount of a gene encoding a protein or polypeptide, as well as an efficient method for producing a protein or polypeptide through use of the modified promoter DNA.

[0011]

In Bacillus subtilis, 17 sigma factors——which are subunits of an RNA polymerase complex——have been identified to be associated with recognition of a promoter sequence. They include SigA (also called a housekeeping sigma factor), which is a primary sigma factor that participates in transcription of a gene which is essential for growth during the vegetative growth period; SigH, SigF, SigE, SigG, and SigK, which control sporulation; SigD, which controls flagellum biogenesis and cell wall lysis; SigL, which controls metabolism of certain amino acids or saccharides; SigB, which controls the ability of adjustment to environmental changes; and a sigma factor named ECF sigma. Respective sigma factors, when bound to an RNA polymerase core complex composed of five subunits (α, β, β', δ, ω) other than a sigma factor, participate in recognition of a promoter sequence in such a manner that a different sigma factor recognizes different promoter sequence, to thereby attain transcription of different genes. This mechanism is

considered to regulate expression, as the situation requires, of approximately 4,100 genes present on the genome.

[0012]

During vegetative growth, through association with an RNA polymerase core complex, SigA is reported to predominantly direct transcription of a gene having a SigA-recognizable promoter, or an operon, and thereafter, during sporulation, when a sigma factor that controls the sporulation process is activated, substitution takes place to replace the sigma factor that is associated with the RNA polymerase core complex, resulting in a relative decrease in the amount of SigA-associated RNA polymerase (J. Bacteriol., 179, 4969 (1999)). Thus, during and after the sporulation stage, the level of transcription from a SigA-recognized promoter is considered to decrease as compared with the level in the vegetative growth stage.

[0013]

Under the above circumstances, the present inventors have found that, through subjecting a DNA fragment having a promoter recognized by SigA which is a sigma factor of *Bacillus subtilis* to base modification through genetic engineering so as to newly construct a sequence recognized by SigE, with the recognition by SigA being maintained, transcription of genes encoding proteins or polypeptides ligated downstream of the modified promoter recognized by SigA and SigE can be enhanced.

[0014]

When the promoter DNA of the present invention is employed, transcription of genes encoding proteins or polypeptides ligated downstream of the promoter DNA can be enhanced considerably as compared with a natural promoter, whereby proteins or polypeptides can be produced efficiently.
[0015]

In the present invention, homology between amino acid sequences and that between nucleic acid sequences are both determined by use of the Lipman-Pearson method (Science, 227, 1435 (1985)). Specifically, calculation is performed by use of a homology analysis program (Search Homology) developed by genetic information processing software Genetyx-Win, (Software Development Co., Ltd.), with ktup (the unit size to be compared, employed as a parameter) being set to 2.
[0016]

It is generally accepted that a sigma factor is bound to a sequence of several bases that is present in the vicinity of a 10-base upstream site or 35-base upstream site from the transcription start point. The sequences corresponding to these sites are called the -10 region and the -35 region, respectively. Moreover, it has been known that, for each sigma factor, common characteristics are shared by the base sequence and the distance between the two regions. Thus, such a sequence is called a consensus sequence, and is considered to form the essential part of the promoter. The consensus sequence of SigA is known to have a -35 region of TTGaca and a -10 region of tgnTAtaat, which is

linked to a site 14 nucleotides downstream from the -35 region (n represents A, G, C, or T, and when nucleotides are shown with upper case letters, the nucleotides are highly conserved, whereas when nucleotides are shown with lower case letters, the nucleotides are not well conserved. See *Bacillus Subtilis* and Its Closest Relatives: From Genes to Cells, Edited by A. L. Sonenshein, American Society for Microbiology, pp289, (2002)). Separately, several consensus sequences recognized by SigA are known to be present in a nucleotide sequence ranging from base Nos. 92 to 552 in SEQ ID NO: 1 and a nucleotide sequence ranging from base Nos. 133 to 589 in SEQ ID NO: 2 (Biosci Biotechnol Biochem. 64, 2281, 2000, Biosci Biotechnol Biochem. 56, 872, (1992)).

[0017]

Therefore, preferred examples of the present nucleotide sequences having a promoter recognized by SigA and nucleotides in the vicinity of the promoter include the following: a nucleotide sequence ranging from base Nos. 92 to 552 in SEQ ID NO: 1; a nucleotide sequence ranging from base Nos. 133 to 589 in SEQ ID NO: 2; or a nucleotide sequence having a homology of 80% or more, preferably 90% or more, more preferably 95% or more, even more preferably 98% or more, to either of these sequences, and having a consensus sequence of SigA and/or promoter functions equivalent to those of the consensus sequence. Preferably, the present nucleotide sequences include a nucleotide sequence having a nucleotide sequence represented by SEQ ID NO: 1; a nucleotide sequence

represented by SEQ ID NO: 2; or a nucleotide sequence having a homology of 90% to either of these sequences, preferably 95% or more, more preferably 98% or more, and having the consensus sequence which is recognized by SigA and/or promoter functions equivalent to those of the consensus sequence. The nucleotide sequences having a nucleotide sequence ranging from base Nos. 92 to 552 in SEQ ID NO: 1 are, in a nucleotide sequence in SEQ ID NO: 1, preferably a 461 to 570 bp consecutive nucleotide sequence having a nucleotide sequence of base Nos. 92 to 552, more preferably a 461 to 520 bp consecutive nucleotide sequence, even more preferably a 461 to 480 bp consecutive nucleotide sequence. The nucleotide sequences having a nucleotide sequence ranging from base Nos. 133 to 589 in SEQ ID NO: 2 are, in a nucleotide sequence represented by SEQ ID NO: 2, preferably a 457 to 610 bp consecutive nucleotide sequence having a nucleotide sequence of base Nos. 133 to 589, more preferably a 457 to 520 bp consecutive nucleotide sequence, even more preferably a 457 to 480 bp consecutive nucleotide sequence.

Here, a nucleotide sequence represented by SEQ ID NO: 1 is present upstream of an alkaline cellulase gene derived from *Bacillus* sp. KSM-S237 (FERM BP-7875), whereas a nucleotide sequence represented by SEQ ID NO: 2 is present upstream of an alkaline cellulase gene derived from *Bacillus* sp. KSM-64 (FERM BP-2886), the two nucleotide sequences having a homology of 95.6%.

[0018]

The promoter DNA of the present invention may be constructed by subjecting the above nucleotide sequences to base modification so as to be recognized not only by SigE but also by SigA. The number of promoters to be constructed may be one, or alternatively two or more.

[0019]

Promoter sequences recognized by SigE are reported to have a nucleotide sequence formed of a -35 region represented by ATAHTT (H denotes A, C, or T) and -10 region represented by CATAYAHT (Y denotes C or T), which is linked to the site 13 or 14 nucleotides downstream from the -35 region, preferably, a nucleotide sequence formed of a -35 region represented by ATATTT and a -10 region represented by CATACAAT, which is linked to the site 13 or 14 nucleotides downstream from the -35 region, and more preferably a nucleotide sequence represented by ATATTTCAAGTAGTAATAACATACAAT (J. Mol. Biol. 327, 945, (2003)). Preferably, such nucleotide sequences are newly constructed.

Preferred promoter DNA in the present invention include a nucleotide sequence represented by SEQ ID NO: 7, which have been produced by modifying a nucleotide sequence represented by SEQ ID NO: 1, and a nucleotide sequence represented by SEQ ID NO: 8, which have been produced by modifying a nucleotide sequence represented by SEQ ID NO: 2.

[0020]

Base modification is performed through insertion of a DNA fragment having a promoter sequence recognized by SigE,

or through deletion, substitution, or insertion of one or more bases. Of these modifications, substitution of one or more bases is preferred. Specifically, a promoter DNA having an inserted promoter sequence recognized by SigE can be constructed as follows. A restriction enzyme recognition site is introduced into any site of a DNA fragment (SEQ ID NO: 1) originating from a region upstream of an alkaline cellulase gene from *Bacillus* sp. KSM-S237 (FERM BP-7875), which gene had been introduced into a plasmid vector by cloning, or of a DNA fragment (SEQ ID NO: 2) derived from a region upstream of an alkaline cellulase gene derived from *Bacillus* sp. KSM-64 (FERM BP-2886) etc., which gene had been introduced into a plasmid vector by cloning, through site-specific mutagenesis such as the Kunkel method (Proc. Natl. Acad. Sci. USA., 82, 488, 1985). Separately, a DNA fragment having the promoter sequence recognized by SigE is prepared through chemical synthesis or a similar method so as to have a restriction enzyme recognition site at each end; and the thus-prepared two fragments, which have been treated with the same restriction enzyme, are ligated with ligase.

[0021]

Alternatively, the above DNA can be constructed through subjecting a potion of a DNA fragment derived from an upstream region of an alkaline cellulase gene represented by SEQ ID NO: 1 or SEQ ID NO: 2 to base substitution through the SOE (splicing by overlap extension)-PCR method (Gene, 77, 51, 1989) or a similar method.

[0022]

Next will be described in more detail a method for newly constructing a promoter (sequence) recognized by SigE through subjecting a potion of a DNA fragment having a nucleotide sequence of SEQ ID NO: 1 to base substitution through the SOE-PCR method.

[0023]

In the first PCR, the following two fragments are prepared: an upstream DNA fragment containing a site having a substituted base at the downstream end, and a downstream DNA fragment containing a site having a substituted base at the upstream end. In this step, above-described DNA fragments are designed so as to anneal with two primers, each of which is respectively designed for downstream of the upstream DNA fragment and upstream of the downstream DNA fragment, for example, and at the same time, the above-described DNA fragments are constructed in a way as to contain the promoter sequence recognized by SigE (Fig. 1).

[0024]

Next, using the two DNA fragments prepared in the first PCR as templates, the second PCR is performed by use of an upstream primer of the upstream DNA fragment and a downstream primer of the downstream DNA fragment. As a result, the downstream end of the upstream DNA fragment anneals with the upstream end of the downstream DNA fragment through the overlapping sequences. The two DNA fragments are ligated through PCR amplification, to thereby obtain a DNA fragment

having newly constructed promoter sequence recognized by SigE at a ligation site (Fig. 1).

[0025]

The thus-constructed promoter DNA is recognized not only by SigE but also by SigA, facilitating transcription, during the sporulation stage, of a gene encoding a protein or polypeptide ligated downstream of the above promoter DNA.

Specifically, when a heterologous protein or polypeptide is produced by use of *Bacillus subtilis* through a recombinant technique, through ligation of the promoter DNA to a site upstream of a gene encoding the target protein or polypeptide, during the vegetative stage, the genes can be transcribed by RNA polymerase bound to SigA, and during the sporulation stage which follows the vegetative stage, the gene can be transcribed by RNA polymerase bound to SigE, thus achieving continued transcription during the sporulation stage. Therefore, when a recombinant *Bacillus subtilis* introduced by a expression vector containing the above promoter DNA is employed, the target proteins or polypeptides can be produced considerably, as compared with *Bacillus subtilis* having a natural promoter other than the newly constructed promoter recognized by SigE.

[0026]

No particular limitation is imposed on the gene encoding the target protein or polypeptide. Examples of the protein and polypeptide include physiologically-active peptides and enzymes for industrial purposes such as

detergents, foods, fibers, feeds, chemicals, medicine, and diagnostic agents. Industrial enzymes may be functionally grouped into oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases/synthetases. Preferably, hydrolases such as cellulase, α-amylase, and protease may be used. Specific examples include cellulase belonging to family 5 in the classification of enzymes which hydrolyze polysaccharides (Biochem. J., 280, 309, (1991)); in particular, cellulase derived from a microorganism, more particularly cellulase derived from the genus *Bacillus*. More specific examples include alkaline cellulase having an amino acid sequence of SEQ ID NO: 4 which is derived from *Bacillus* sp. KSM-S237 (FERM BP-7875), alkaline cellulase having an amino acid sequence of SEQ ID NO: 6 which is derived from *Bacillus* sp. KSM-64 (FERM BP-2886), and cellulase which has another amino acid sequence having a homology of 70% or more to said amino acid sequence, preferably 80% or more, more preferably 90% or more, further preferably 95% or more, or still further preferably 98% or more.

[0027]

Specific examples of α-amylase include α-amylase derived from a microorganism, preferably liquefied amylase derived from the genus *Bacillus*. More specific examples include alkaline amylase having an amino acid sequence of SEQ ID NO: 14 which is derived from *Bacillus* sp. KSM-K38 (FERM BP-6946), and amylase which has another amino-acid sequence having a homology of 70% or more to said amino-acid sequence,

preferably 80% or more, more preferably 90% or more, further preferably 95% or more, even more preferably 98% or more. Specific examples of protease include serine protease and metalloprotease which are derived from microorganisms, particularly those belonging to the genus *Bacillus*.

[0028]

In addition to the promoter DNA of the present invention, preferably, regulatory regions related to secretion or translation of the target protein or polypeptide; i.e., a ribosome-bound site (SD sequence), a translation initiation region including the initiation codon, and a secretion signal peptide region, are properly ligated to the gene of the target protein or polypeptide. In one preferred example, a transcription initiation regulatory region, a translation initiation region, and a secretion signal peptide region of a cellulase gene derived from a microorganism belonging to the genus *Bacillus* disclosed in, for example, JP-A-2000-210081 or JP-A-1992-190793; i.e., a cellulase gene derived from KSM-S237 (FERM BP-7875) or KSM-64 (FERM BP-2886), is properly ligated to a structural gene of the target protein or polypeptide. More specifically, preferred DNA fragments to be ligated include a nucleotide sequence ranging from base Nos. 563 to 659 of SEQ ID NO: 3; a nucleotide sequence ranging from base Nos. 600 to 696 of a cellulase gene of SEQ ID NO: 5; a DNA fragment having a nucleotide sequence having a homology of 70% or more to any one of said nucleotide sequences, preferably 80% or more,

more preferably 90% or more, further preferably 95% or more, further more preferably 98% or more; or a DNA fragment having a nucleotide sequence lacking a portion of any one of said nucleotide sequences. Preferably, one of these DNA fragments is properly ligated to a structural gene of the target protein or polypeptide.

[0029]

Productivity of the target protein or polypeptide can be enhanced by use of a recombinant microorganism, which is constructed by transferring an expression vector into a *Bacillus subtilis* cell through a conventional transformation technique. The expression vector is produced by ligating the promoter DNA of the present invention upstream of a DNA fragment containing the above gene encoding the target protein or polypeptide, and inserting the thus-ligated DNA fragment into an appropriate vector. Alternatively, the promoter DNA of the present invention is ligated to an appropriate region which is homologous with the genome of *Bacillus subtilis*, to thereby prepare a DNA fragment. The DNA fragment is inserted directly into the genome of *Bacillus subtilis*, to thereby construct a recombinant cell strain. Productivity of the target protein or polypeptide may be enhanced by use of the recombinant cell strain.

[0030]

The target protein or polypeptide obtained by use of the promoter DNA of the present invention may be produced in such a manner that the above recombinant microorganism is

inoculated onto a culture medium containing assimilable carbon sources and nitrogen sources, and other essential components; the microorganism is cultured through a conventional microorganism culturing method; and subsequently, protein or polypeptide is collected and purified.

[0031]

Next, a method for constructing a DNA fragment of the present invention and a method for producing cellulase through a recombinant technique with the DNA fragment will be described in detail by way of Examples.

Examples

[0032]

Example 1 Construction of promoter (sequence) recognized by SigE in upstream region of alkaline cellulase gene

A promoter recognized by SigE was introduced into an upstream region of an alkaline cellulase gene in accordance with the procedure as shown in Fig. 2. Specifically, a recombinant plasmid pHY-S237, serving as a template, and a primer set of 237UB1 and EP1UPr shown in Table 1 were employed, to thereby prepare an upstream region of an alkaline cellulase gene; i.e., a 0.4 kb fragment (A). The recombinant plasmid pHY-S237 was prepared by inserting a DNA fragment (3.1 kb) encoding an alkaline cellulase gene (JP-A-2000-210081) derived from *Bacillus* sp. KSM-S237 (FERM BP-7875) into the restriction enzyme *Bam*HI cleavage site of a shuttle vector pHY300PLK. Similarly, a primer set of EP1DNf

and S237RV shown in Table 1 was employed, to thereby prepare a downstream region of an alkaline cellulase gene; i.e., a 2.7 kb fragment (B). Subsequently, SOE-PCR was performed by use of a primer set of 237UB1 and S237RV shown in Table 1 and the two fragments (A) and (B) as a template mixture, to thereby produce a 3.1 kb DNA fragment (C) in which the fragments (A) and (B) were ligated in this sequence. Primers EP1UPr and EP1DNf had been subjected to base substitution treatment, and, as shown in Fig. 2, the DNA fragment (C) contained a newly constructed promoter (sequence) recognized by SigE in a region about 150 bp upstream from the translation initiation site of the alkaline cellulase. The thus-obtained 3.1 kb DNA fragment (C) was inserted into the *Sma*I restriction enzyme cleavage site of a shuttle vector pHY300PLK, to thereby construct a recombinant plasmid pHY-S237EP1. Separately, the recombinant plasmid pHY-S237, serving as a template, and a primer set of 237UB1 and S237RV shown in Table 1 were employed, to thereby prepare a 3.1 kb fragment (D) containing a full length alkaline cellulase gene. Subsequently, the 3.1 kb fragment (D) was inserted into the *Sma*I restriction enzyme cleavage site of a shuttle vector pHY300PLK, to thereby construct a recombinant plasmid pHY-S237W.

[0033]

Example 2 Evaluation of alkaline cellulase production (secretion) performance

The recombinant plasmid pHY-S237EP1 obtained in Example

1 and a recombinant plasmid pHY-S237W serving as a control were individually introduced to cells of *Bacillus subtilis* 168 through the protoplast transformation method. The cells were shake-cultured in LB medium (10 mL) overnight at 37°C. The culture broth (0.05 mL) was inoculated to a 2 × L-maltose medium (50 mL) (2% tryptone, 1% yeast extract, 1% NaCl, 7.5% maltose, 7.5 ppm manganese sulfate 4-5 hydrate, and 15 ppm tetracycline), followed by shake-culturing at 30°C for three days. After completion of culturing, cells were removed through centrifugation, and alkaline cellulase activity of the supernatant obtained from the culture was determined for calculation of the amount of the alkaline cellulase secreted from the cells during culturing; i.e., the amount of the extracellularly produced alkaline cellulase. As is clear from Table 2, more effective production, or secretion, of alkaline cellulase has been confirmed in the case where pHY-S237EP1 was employed as a recombinant plasmid, as compared with the control pHY-S237W (wild type). Thus, use of pHY-S237EP1 enhances cellulase production or secretion, conceivably because transcription from the newly constructed promoter recognized by SigE was added to transcription from a promoter recognized by SigA.

[0034]

[Table 1]

Primer	Nucleotide sequence	SEQ ID NO.
237UB1	TTGCGGATCCAACAGGCTTATATTTAGAGGAAATTTC	9
EP1UPr	GTATGTTATTACTACTTGAAATATTCTACCCCCCTTCCTA	10
EP1DNf	ATATTTCAAGTAGTAATAACATACAATACTTATAAGTTG	11
S237RV	TCGCTACCCTTTTATTATCG	12

[0035]

[Table 2]

Recombinant plasmid	Amount of produced (secreted) alkaline cellulase (relative value)
pHY-S237W (wild type)	100
pHY-S237EP1	165

[0036]

Example 3 Alkaline amylase production performance of upstream region of alkaline cellulase gene containing promoter (sequence) recognized by SigE

The plasmid pHY-S237EP1 constructed in Example 1, serving as a template, and a primer set of S237ppp-F2 (*Bam*HI) and S237ppp-R2 (ALAA) shown in Table 3 were employed, to thereby amplify through PCR a 0.6 kb DNA fragment (E) containing a region encoding a secretory signal sequence and a promoter region of the alkaline cellulase to which an SigE-recognized promoter (sequence) has been transferred. Separately, PCR was performed using a genome DNA sample extracted from *Bacillus* sp. KSM-K38 (FERM BP-6946) as a template, and a primer set of K38matu-F2 (ALAA) and SP64K38-R (*Xba*I) shown in Table 3, whereby a 1.5 kb DNA fragment (F) encoding alkaline amylase (Appl. Environ. Microbiol., 67, 1744, (2001)) having an amino acid sequence of SEQ ID NO: 14

was amplified. Subsequently, SOE-PCR was performed by use of a primer set of S237ppp-F2 (*Bam*HI) and SP64K38-R (*Xba*I) shown in Table 3 and the thus-obtained two fragments (E) and (F) (in a mixture form) as templates, to thereby produce a 2.1 kb DNA fragment (G) in which an alkaline amylase gene was ligated downstream of the region encoding a secretory signal sequence followed by the promoter region, containing a promoter (sequence) recognized by SigE, of an alkaline cellulase gene. The thus-produced 2.2 kb DNA fragment (G) was inserted into the *Bam*HI-*Xba*I restriction enzyme cleavage site of a shuttle vector pHY300PLK (yakult), to thereby construct a recombinant plasmid pHY-K38 (S237ps) EP1. Separately, the above procedure was repeated, except that the plasmid pHY-S237W constructed in Example 1 was employed instead of the template which had been employed for amplification of the aforementioned 0.6 kb DNA fragment (E), to thereby construct a recombinant plasmid pHY-K38 (S237ps) W.

The thus-constructed plasmid pHY-K38 (S237ps) EP1 and pHY-K38 (S237ps) W (which serves as a control) were individually introduced to cells of *Bacillus subtilis* 168 through the protoplast transformation method. The cells were shake-cultured for five days, and other conditions were the same as employed in Example 2. After completion of culturing, cells were removed through centrifugation, and alkaline amylase activity of the supernatant obtained from the culture was determined for calculation of the amount of the amylase secreted from the cells during culturing; i.e., the amount of

the extracellularly produced amylase. As is clear from Table 4, more effective production, or secretion, of alkaline amylase has been confirmed in the case where pHY-K38 (S237ps) EP1 was employed as a recombinant plasmid, as compared with the control pHY-K38 (S237ps) W (wild type). Thus, it was revealed that the upstream region, containing a promoter (sequence) recognized by SigE, of an alkaline cellulase gene were employed effectively in producing a variety of proteins or polypeptides.

[Table 3]

Primer	Nucleotide sequence	SEQ ID NO.
S237ppp-F2(BamHI)	CCCGGATCCAACAGGCTTATATTTA	15
S237ppp-R2(ALAA)	TTCAATCCATCTGCTGCAAGAGCTGCCGG	16
K38matu-F2(ALAA)	GCTCTTGCAGCAGATGGATTGAACGGTACG	17
SP64K38-R(XbaI)	TTGGTCTAGACCCCAAGCTTCAAAGTCGTA	18

[Table 4]

Recombinant plasmid	Amount of produced (secreted) alkaline amylase (relative value)
pHY-K38 (S237ps) W (wild type)	100
pHY-K38 (S237ps) EP1	143